A technique for examination of diagnostic characters of penicillate millipedes

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ABSTRACT

We describe a simple method for examination of penicillate millipedes. The internal tissues are dissolved and the stain Fast Green used to give excellent contrast for dissection and to provide clear visualisation of characters necessary for identification of both freshly collected and museum specimens. ❐ *Histology, Fast Green stain, Diplopoda, Penicillata, Polyxenida.*

Millipedes in the subclass Penicillata, order Polyxenida have been overlooked in the study of terrestrial invertebrate fauna in Australia. Penicillates are commonly known as bristly, dwarf or pincushion millipedes. They differ from other millipedes in that adults are less than 5 mm in length, the cuticle is unmineralised and the animals are covered in bristles or trichomes (Hopkin & Read 1992). Due to their small size and soft structure, penicillates need to be specially prepared for high-power microscopic examination in order to clearly view their diagnostic characters. Diagnostic features used include insertion patterns of the cephalic, tergal and caudal trichomes; number and nature of antennal and leg sensilla; details of the mouthparts (labrum and gnathochilarium); and number of ocelli (Nguyen Duy-Jacquemin 2006; Short & Huynh 2006). No information is available on histological techniques used to examine penicillates other than preparation of whole mounts. This paper describes the technique we have developed to make permanent mounts using the stain Fast Green, a stain more commonly used in botanical microscopy (Ruzin 1999) and illustrates a selection of the characters used in identification of genera and species. The method has been modified from that described by Upton (1991) for mounting small arthropods for microscopic examination.

MATERIALS AND METHOD

Due to their small size penicillate millipedes are most commonly collected from bark and litter samples using Tullgren funnel extractions and stored in 70-80% ethanol. Selected specimens at least 2 mm in size are then prepared for microscopic examination as described below:

1. Removal and mounting of trichomes: This allows the clearest view of the pattern of trichome insertion points. Place a specimen in a drop of 100% isopropanol on a glass slide and remove trichomes with fine forceps and a microprobe. Gently disperse to separate them and after the isopropanol has evaporated cover the trichomes with a drop of DPX mounting medium and cover-slip.
2. Maceration and clearing: Body contents are digested while retaining details of the exoskeleton including sensory hairs and colxal gland openings. Place previously preserved

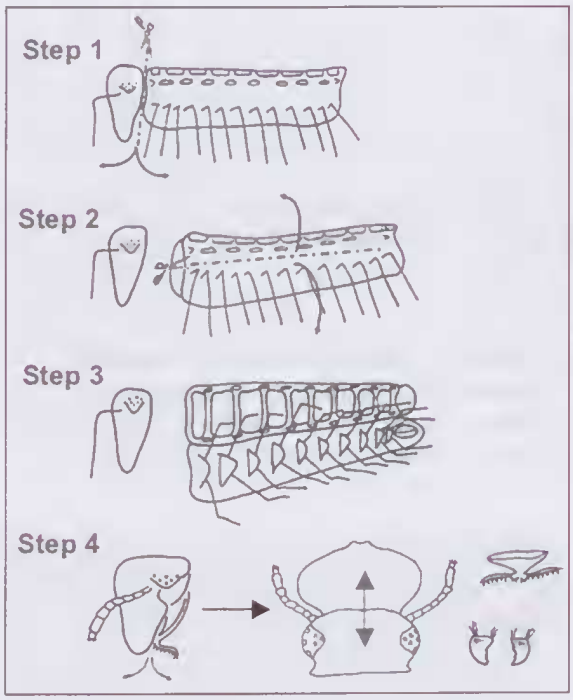

FIG. 1. Technique for opening up exoskeleton of penicillate millipedes in order to remove contents and prepare exoskeleton for staining.

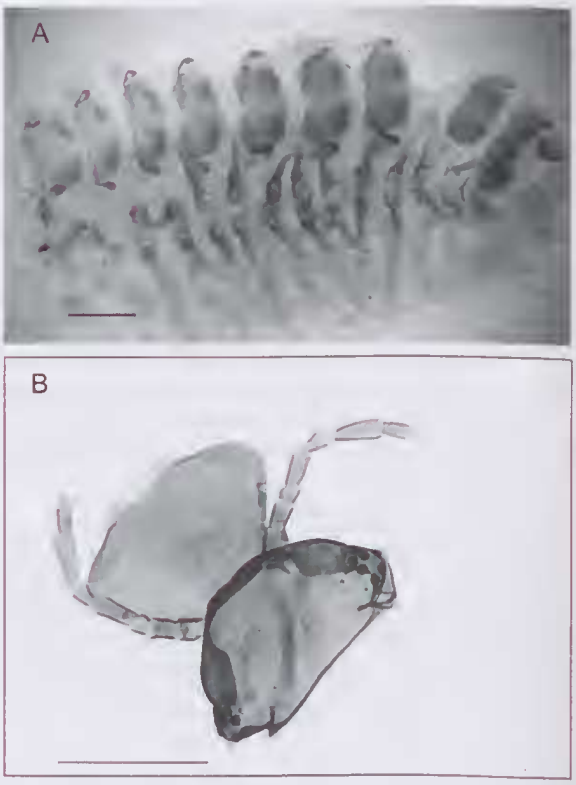

FIG. 2. Exoskeleton of body (A) and head (B) after slide preparation showing both dorsal and ventral features. Scale bars = 500μm.

specimens in an Eppendorf tube with 15% potassium hydroxide and heat gently in a water-bath for 2 minutes at 80°C followed by rinsing in distilled water. Damaged specimens or those preserved for many decades need just 1 minute in potassium hydroxide, while fresh specimens require a longer period of up to 12 hours immersion.

3. Dehydration and staining: Rinse the cleared specimen in water and then place into 20% acetic acid for 2 minutes to neutralise, followed by dehydration through a series of ethanol solutions from 70%, 80%, 90% to 100% (2 minutes per solution), followed by 2 minutes in 1% Fast Green in 100% ethanol. Return the specimen to 100% ethanol to remove excess stain.
4. Cleaning and dissection: The cleared and stained millipede is opened up using microprobes and micro-scissors in a series of steps as illustrated in Fig. 1, followed by removal of body contents. The Head is detached from the body. Removal of contents and opening out of the body and head are required to facilitate viewing of morphological characters of the exoskeleton.
5. Re-staining and mounting: Return the head and body to 1% Fast Green solution in 100% ethanol for 2 minutes followed by 100% isopropanol for 1 minute and finally into xylene for at least 1 minute. Specimens can be left in xylene until ready to mount. Mount

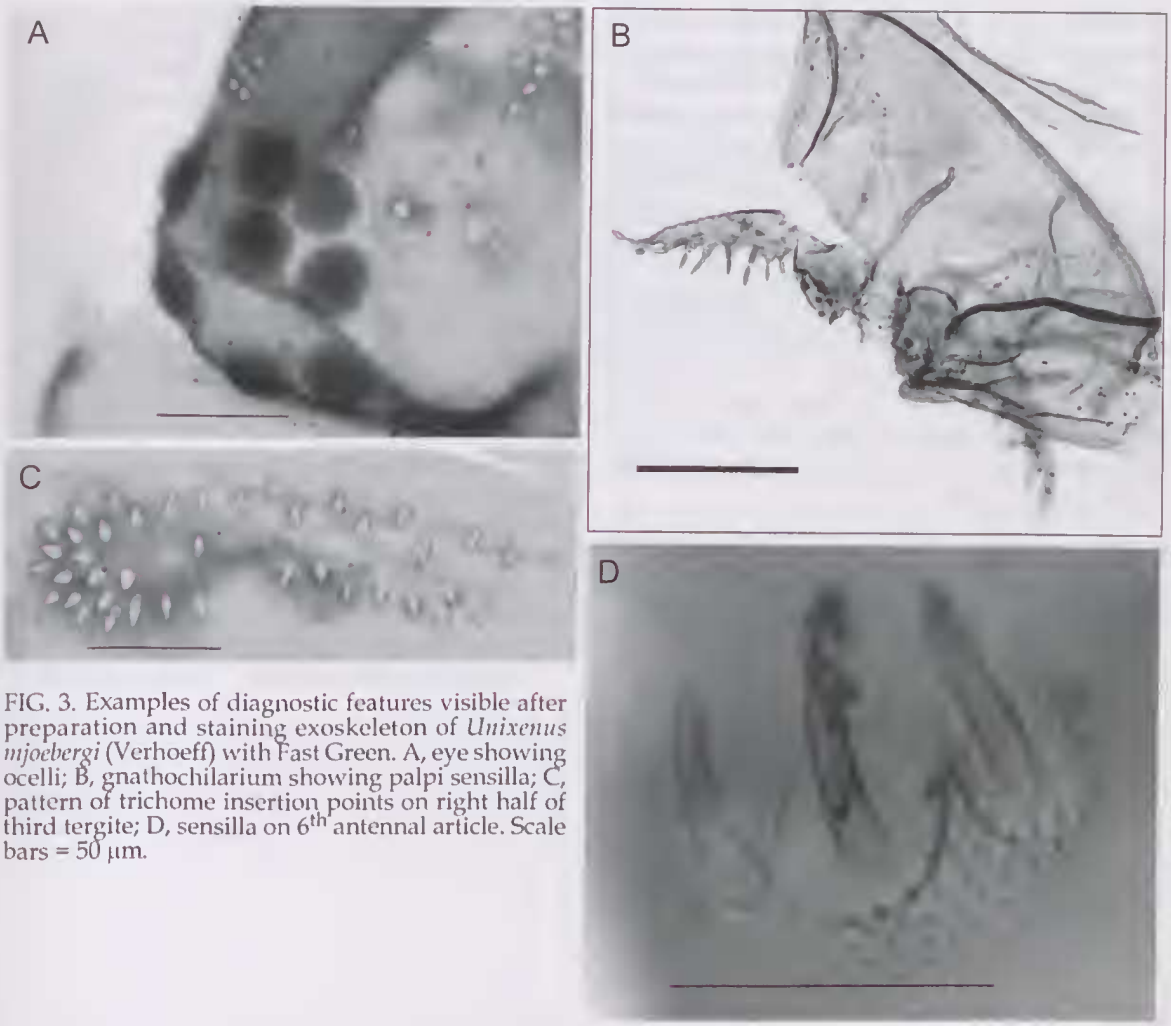

FIG. 3. Examples of diagnostic features visible after preparation and staining exoskeleton of *Unixenus mjoebergi* (Verhoeff) with Fast Green. A, eye showing ocelli; B, gnathochilarium showing palpi sensilla; C, pattern of trichome insertion points on right half of third tergite; D, sensilla on 6th antennal article. Scale bars = 50 μm.

the head and body separately in a drop of xylene on a glass slide followed by a drop of mounting medium DPX. DPX is preferred as it dries faster than Canada Balsam or Euparal. When mounting the head, tease apart the antennae and mouthparts with microprobes so that all relevant diagnostic features are clearly visible. When mounting the body, arrange the split body so that both dorsal and ventral surfaces are in the same plane for ease of viewing.

Images were taken using a Nikon Coolpix 4500 digital camera with an Olympus Vanox compound microscope.

RESULTS AND DISCUSSION

The body and head after preparation and staining are illustrated in Fig. 2. Having a permanent preparation of both dorsal and ventral features in the one field of view, enables efficient examination and documentation of diagnostic

features. Fig. 3 illustrates a selection of these features. The method provides clearer views of features in comparison with a whole mount in which the gut contents often obscure important features such as the pattern of tergal trichome insertions. A further advantage is that staining prior to dissection enables the specimen to be more easily visualised for dissection after maceration renders penicillates transparent and hard to manipulate. As well only one specimen is required to view all features. This is an important factor when only a small number of specimens is available. A final advantage of the technique is reduction of the depth of field of the preparation with the body opened out and the contents removed.

We have used the method successfully with specimens from the penicillate families Synxenidae, Polyxenidae and Lophoproctidae including museum specimens lacking any colouration after decades in preservative.

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